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Original Article

Use of Hydrogen Peroxide (H_2O_2) in raw cow's milk preservation

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ABSTRACT

Objective: Hydrogen peroxide (H_2O_2) was used for the activation of lactoperoxidase system on preservation of milk.

Materials and methods: Milk samples were collected from Bangladesh Agricultural University dairy farm. The collected milk samples were added with 0.02, 0.04, 0.06, 0.08, 0.10, 0.12 and 0.14% of 10% H_2O_2 along with control one. All milk samples were kept at room temperature (28-31^{0}C). Flavor, clot on boiling (COB), acidity %, and methylene blue reduction (MBR) test were observed every one hour interval.

Results: The milk samples were acceptable in terms of flavor up to 18 h at 0.14% H_2O_2. Similarly, this milk sample took maximum time (19 h) to give COB positive test. Acidity % was within normal range for 0.02, 0.04, 0.06, 0.08, 0.10, 0.12 and 0.14% H_2O_2 treated milk samples up to 8, 9, 11, 12, 12, 14, 17 and 18 h, respectively. Significant difference (P<0.01) was found among the levels of H_2O_2 in acidity development at every observation hour except 0 hour. In MBR test, control sample was decolorized at 7 h and H_2O_2 treated milk samples were decolorized after 8 to 13 h depending on the level of added H_2O_2.

Conclusion: Based on the results, it may be recommended that 0.14% H_2O_2 is suitable to extend the milk shelf-life where milk cooling facilities are not available.

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KEYWORDS

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INTRODUCTION

Milk contains many essential nutrients, such as carbohydrates, proteins, lipids, minerals and vitamins and therefore, acts as an ideal medium for rapid proliferation of harmful microorganisms (Saha et al., 2003). Lactoperoxidase (LP) is a glycoprotein that presents by nature in colostrums and milk (Kussendrager and van Hooijdonk, 2000; Conner et al., 2002). This LP system has been proven to be effective against both gram-positive and gram-negative bacteria (Naidu, 2000; Marks et al., 2001). LP system becomes active with the presence of hydrogen peroxide and thiocyanate. Lactoperoxidase system has been considered as an important side in defense mechanism in mammals (Boots and Floris, 2006). Lactoperoxidase is copious in raw milk, whereas, thiocyanate and hydrogen peroxide are present at low concentrations and can be depleted.

The antimicrobial activity of LP is exerted through the oxidation of thiocyanate (SCN^-) by hydrogen peroxide producing oxidation products as hypothiocyanite ($OSCN^-$) (Carlsson et al., 1983; Le Nguyen et al., 2005) and hypthiocyanous acid (HOSCN), which show antimicrobial action (Sermon et al., 2005). Lactoperoxidase catalyzes the chemical reaction in milk of inorganic and organic substrates with the help of H_2O_2 (Al-Baarri et al., 2011). To activate LP system, H_2O_2 is recommended by FAO/WHO as a standard method for inhibiting bacterial growth in raw milk (CAC, 1991). Hydrogen peroxide is the accepted chemical to activate LP system in milk to prevent bacterial proliferation (Ozer et al., 2003). Hydrogen peroxide has toxicant effect when exposed to mammalian cells. But mammalian cells can be insured from this toxicity in the presence of LP and SCN^-, if low concentrations of H_2O_2 are used (Pruitt and Kamau, 1991).

In Bangladesh, due to environmental conditions it is very difficult to preserve milk without applying any technique. Lambert (2001) stated that cooling is the most feasible method to save milk from bacterial deterioration. There may have lot of chances to spoil the milk during transportation due to lack of cooling facilities, unhygienic milk production practices at farm level and improper transport facilities (El Zubeir et al., 2010). Seifu et al. (2005) stated that LP system is the most effective technique of milk preservation where refrigeration facilities are not easy to manage. In Bangladesh, maintaining cooling chain is inaccessible due to inadequate electricity supply and extra cost involvement. Saha et al. (2003) and Rokhsana et al. (2007) claimed a positive influence of adding H_2O_2 to milk with respect to preservation. Rokhsana et al. (2007) were able to extend the self-life of milk up to 11 h by adding 0.05% H_2O_2 at 20^0C though the concentration/grade/purity of H_2O_2 were not declared. On the other hand, Saha et al. (2003) concluded that 0.04 to 0.05% H_2O_2 (30 w/v) was enough to extend the self-life of milk up to 24 h. However, there are ample opportunities to generate new scientific data in this domain. Different doses of 10% H_2O_2 were used in this experiment to monitor the shelf-life of raw milk with a focus to bacterial growth. For this purpose, changes in flavor and acidity, milk clotting and methylene blue reduction time were recorded.

MATERIALS AND METHODS

Place of experiment: The experiment was conducted at Dairy Chemistry Laboratory, Department of Dairy Science, Bangladesh Agricultural University (BAU), Mymensingh.

Source of milk: Raw cow's milk was collected from BAU dairy farm. Pooled milk samples of ten cows (Holstein-friesian cross and Jersey cross) were collected and immediately used for the study. All hygienic measures were followed during sampling.

Dilution of hydrogen peroxide (H_2O_2): Hydrogen peroxide (35% extra pure, MERCK, Germany) was diluted at the ratio of 1:2.5 (H_2O_2: Distilled water) before adding with milk to reduce the concentration of food grade H_2O_2 from 35% to 10%. Immediate after dilution, H_2O_2 was added with previously measured milk.

Preparation of milk samples: Milk samples were divided into eight equal parts. Out of the eight parts, one part was kept as untreated milk (control) and remaining seven parts were treated with 0.02, 0.04, 0.06, 0.08, 0.10, 0.12 and 0.14% of 10% H_2O_2. Milk samples were kept in glass beaker wrapped with aluminum foil (DIAMOND Aluminum Foil, 2001 Reynolds Consumer Products, A Business of Alcoa Inc., Richmond, Virginia 23261, USA) at room temperature (28-31^0C) to monitor shelf-life. After treating milk with different level of H_2O_2, following tests were conducted for all eight milk samples at every one hour interval until spoilage of milk.

Changes in flavor: Appearance of unpleasant (slight sour) flavor was detected by organoleptic test.

Clot on boiling (COB) test: COB (AOAC, 1990) test was performed to detect acid milk (pH<5.8). Milk sample (3 mL) was boiled in test tube over sprit lamp. If there was clotting/coagulation/precipitation, the milk sample was failed the test due to higher developed acidity.

Acidity test: Acidity test (AOAC, 1990) was employed to ascertain the extent of developed acidity due to bacterial

fermentation of milk sugar. In acidity test, 18 g milk sample was titrated against 0.1N NaOH with phenolphthalein as indicator.

Methylene blue reduction (MBR) test: MBR test is an indirect method of estimating bacterial population (Bapat et al., 2006; Nandy and Venkatesh, 2010). Methylene blue is a redox indicator that loses its color under the absence of oxygen. Oxygen removal from milk due bacterial consumption and the formation of reducing substances during bacterial metabolism cause the color to disappear. 1 mL methylene blue was added to 10 mL of milk and incubated at 36^{0}C. Reduction times were recorded and the samples were graded (Excellent, decolorized after 8 h; Good, decolorized within 6 to 8 h; Fair, decolorized within 2 to 6 h; Poor, decolorized within 30 min to 2 h; and Very Poor, decolorized below 30 min).

Statistical methods: Completely Randomized Design (CRD) was applied to find out statistical difference by using SPSS (Version 17.0 Chicago, SPSS Inc.).

RESULTS AND DISCUSSION

Changes in flavor: At initial stage, milk samples were appeared with pleasant flavor. It was found that addition of 0.14% H_2O_2 to the milk extended the shelf-life by 10 h compared to that of the control milk sample. The extension of shelf-life followed the ascending order of the level of H_2O_2 added (**Table 1**). The finding suggests that H_2O_2 stunts the bacterial fermentation of milk sugar resulting delayed sour flavor development. Saha et al. (2003) used 30% H_2O_2 where milk sample was acceptable up to 27 h treated with 0.06% H_2O_2. This difference with our experiment may be due to concentration of H_2O_2. Flavor deterioration is mainly related with microbial fermentation which prohibited by LP (Garcia-Graells et al., 2000).

Table 1. Flavor observation of control and different levels of H_2O_2 treated milk samples.

Level of H_2O_2 (%)	Hours of developing sour flavor
Control	9
0.02	11
0.04	12
0.06	13
0.08	14
0.10	15
0.12	18
0.14	19

Clot on boiling (COB) test: The result of COB test is shown in **Table 2**. From the results, it is evident that untreated milk sample clotted 2 h earlier than 0.02% H_2O_2 treated milk sample. The appearance of milk clotting for remaining samples in COB test followed an increasing trend with the concentration of H_2O_2. This was due to gradual delaying of lactic acid development in increased level of H_2O_2 treated milk samples.

It is mentioned by Naidu (2000) and Marks et al. (2001) that H_2O_2 inhibits the growth of acid producing bacteria in milk. Lactoperoxidase system impedes the developed acidity by retarding bacterial growth (Saad et al., 2013). Lin et al. (2000) reported that different levels of H_2O_2 increased the shelf-life of milk.

Acidity test: The acidity percentage of control and H_2O_2 treated milk samples are shown in **Table 3**. The apparent acidity range (0.14-0.17%) were recorded for control, 0.02, 0.04, 0.06, 0.08, 0.10, 0.12 and 0.14% H_2O_2 treated milk samples up to 8, 9, 11, 12, 12, 14, 17 and 18 h,

Table 2. Positive COB time of control and different level of H_2O_2 treated milk samples.

Hours	Level of H_2O_2							
	Control	0.02%	0.04%	0.06%	0.08%	0.10%	0.12%	0.14%
0	–	–	–	–	–	–	–	
4	–	–	–	–	–	–	–	–
6	–	–	–	–	–	–	–	–
8	–	–	–	–	–	–	–	–
9	–	–	–	–	–	–	–	–
10	+	–	–	–	–	–	–	–
11	+	–	–	–	–	–	–	–
12	+	+	–	–	–	–	–	–
13	+	+	+	–	–	–	–	–
14	+	+	+	+	–	–	–	–
15	+	+	+	+	+	–	–	–
16	+	+	+	+	+	+	–	–
17	+	+	+	+	+	+	–	–
18	+	+	+	+	+	+	+	–
19	+	+	+	+	+	+	+	+

Table 3. Average acidity of control and different level of H_2O_2 treated milk samples.

Hour	Level of H_2O_2								*P* value
	Control	0.02%	0.04%	0.06%	0.08%	0.10%	0.12%	0.14%	
0	$0.140^{a}\pm0.000$	$0.140^{a}\pm0.000$	$0.140^{a}\pm0.000$	$0.140^{a}\pm0.000$	$0.140^{a}\pm0.000$	$0.140^{a}\pm0.000$	$0.140^{a}\pm0.000$	$0.140^{a}\pm0.000$	NS
4	$0.148^{a}\pm0.002$	$0.148^{a}\pm0.002$	$0.146^{a}\pm0.005$	$0.143^{ab}\pm0.002$	$0.140^{b}\pm0.000$	$0.140^{b}\pm0.000$	$0.140^{b}\pm0.000$	$0.140^{b}\pm0.000$	0.001
6	$0.160^{a}\pm0.005$	$0.155^{ab}\pm0.005$	$0.153^{b}\pm0.002$	$0.146^{c}\pm0.002$	$0.146^{c}\pm0.002$	$0.145^{c}\pm0.000$	$0.145^{c}\pm0.000$	$0.141^{c}\pm0.002$	0.000
8	$0.170^{a}\pm0.002$	$0.161^{a}\pm0.007$	$0.155^{bc}\pm0.005$	$0.150^{c}\pm0.000$	$0.148^{c}\pm0.002$	$0.148^{c}\pm0.002$	$0.148^{c}\pm0.002$	$0.148^{c}\pm0.002$	0.000
9	$0.181^{a}\pm0.002$	$0.166^{b}\pm0.011$	$0.161^{bc}\pm0.002$	$0.155^{cd}\pm0.000$	$0.150^{d}\pm0.005$	$0.151^{d}\pm0.002$	$0.148^{d}\pm0.002$	$0.148^{d}\pm0.002$	0.000
10	$0.193^{a}\pm.005$	$0.176^{b}\pm0.007$	$0.166^{c}\pm0.002$	$0.160^{cd}\pm0.000$	$0.155^{de}\pm0.000$	$0.153^{de}\pm0.005$	$0.148^{e}\pm0.002$	$0.148^{e}\pm0.002$	0.000
11	$0.205^{a}\pm0.005$	$0.186^{b}\pm0.007$	$0.170^{c}\pm0.005$	$0.165^{cd}\pm0.000$	$0.161^{de}\pm0.002$	$0.155^{ef}\pm0.005$	$0.151^{f}\pm0.002$	$0.151^{f}\pm0.002$	0.000
12	$0.255^{a}\pm0.008$	$0.196^{b}\pm0.007$	$0.178^{c}\pm0.007$	$0.170c^{d}\pm0.000$	$0.170^{cd}\pm0.005$	$0.161d^{e}\pm0.005$	$0.156^{e}\pm0.007$	$0.151^{e}\pm0.002$	0.000
13	$0.340a\pm0.017$	$0.215^{b}\pm0.013$	$0.191^{c}\pm0.012$	$0.181^{cd}\pm0.005$	$0.175^{cde}\pm0.005$	$0.168^{de}\pm0.002$	$0.160^{e}\pm0.005$	$0.160^{e}\pm0.005$	0.000
14	$0.405^{a}\pm0.032$	$0.258^{b}\pm0.007$	$0.222^{c}\pm0.000$	$0.193^{d}\pm0.010$	$0.180^{de}\pm0.005$	$0.168^{e}\pm0.002$	$0.163^{e}\pm0.002$	$0.163^{e}\pm0.007$	0.000
15	$0.453^{a}\pm0.058$	$0.311^{b}\pm0.041$	$0.263^{c}\pm0.002$	$0.225^{cd}\pm0.020$	$0.191^{de}\pm0.002$	$0.173^{e}\pm0.002$	$0.165^{e}\pm0.005$	$0.166^{e}\pm0.005$	0.000
16	$0.493^{a}\pm0.061$	$0.368^{b}\pm0.045$	$0.285^{c}\pm0.018$	$0.251^{cd}\pm0.027$	$0.208^{de}\pm0.010$	$0.178^{e}\pm0.002$	$0.168^{e}\pm0.002$	$0.167^{e}\pm0.005$	0.000
17	$0.531^{a}\pm0.064$	$0.417^{b}\pm0.059$	$0.316^{c}\pm0.015$	$0.268^{cd}\pm0.037$	$0.235^{de}\pm0.021$	$0.188^{e}\pm0.010$	$0.170^{e}\pm0.002$	$0.170^{e}\pm0.002$	0.000
18	$0.555^{a}\pm0.058$	$0.465^{b}\pm0.040$	$0.343^{c}\pm0.032$	$0.310^{cd}\pm0.020$	$0.266^{d}\pm0.017$	$0.205^{e}\pm0.013$	$0.193^{e}\pm0.007$	$0.171^{e}\pm0.002$	0.000
19	$0.588^{a}\pm0.048$	$0.494^{b}\pm0.047$	$0.386^{c}\pm0.030$	$0.353^{cd}\pm0.015$	$0.303^{d}\pm0.019$	$0.235^{e}\pm0.021$	$0.206^{e}\pm0.012$	$0.183^{e}\pm0.002$	0.000

Means with different superscripts in the same row differred significantly.

Table 4. Observation of Methylene Blue Reduction (MBR) test for various milk samples.

Concentrations of H_2O_2	Observation time (h)														
	8.00 am	8.30 am	9.0 am	10.0 am	11.0 am	12.0 pm	1.0 pm	2.0 pm	3.0 pm	4.0 pm	5.0 pm	6.0 pm	7.0 pm	8.0 pm	9.0 pm
Control	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+
0.02%	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+
0.04%	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+
0.06%	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+
0.08%	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+
0.10%	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+
0.12%	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+
0.14%	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+

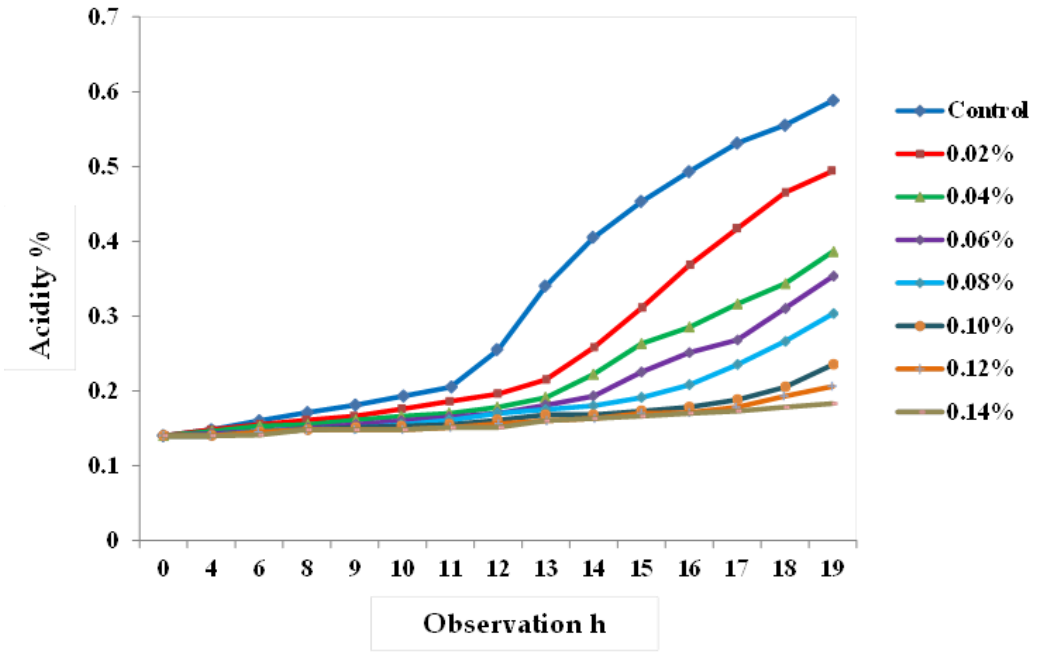

Figure 1. Changes in acidity of control and different level of H_2O_2 treated milk samples.

respectively. The acidity of all milk samples were studied up to 19 h until last milk sample (0.14%) was clotted. In **Figure 1**, acidity of control milk sample was increased steadily up to 0.588% at 19th hour in which point it was one-third in case of 0.14% H_2O_2 treated milk sample. Acidity test revealed that H_2O_2 prolonged shelf-life of milk successfully. Significantly (P<0.0) lowest acidity % was found in each observation in the samples added different level of H_2O_2. The time depends on level of H_2O_2 used as preservative. The result of acidity test agrees in general with the report of Saha et al. (2003). In their experiment they used 0.01, 0.02, 0.03, 0.04, 0.05, and 0.06% H_2O_2 and recorded average acidity % up to 32 h.

The outcome of this study is very close to that reported by Kang et al. (1983) where 0.02% H_2O_2 was used to protect milk from microbial fermentation for at least 12 h. El Zubeir et al. (2010) reported that initial acidity of bovine milk was 0.209% and remained unchanged (0.209%) even after 9 days of storage when treated with LP system. Ambadkar and Lembhe (1991) have shown that 300 ppm H_2O_2 significantly increased shelf life of milk for 18 h. Saad et al. (2013) found significant (P≤0.001) difference in acidity among control and LP enzyme treated milk samples where they reported that LP system helped in reduction acidity in milk until 9 days in the refrigerator storage. Haddadin et al. (1996) used different ratio of SCN^- and H_2O_2 and reported acidities were unchanged (P<0.01) for 4 days when milk samples stored at 4°C. Organic acids mostly lactic acid is the result of homofermentation of milk sugar. H_2O_2 retard bacterial growth that is responsible for acid production in raw milk.

Methylene blue reduction (MBR) test: Good milk needs to decolorize in MBR test about 6 to 8 h where in this study the control sample was decolorized at 7.00 h which proved as good quality shown in **Table 4**. H_2O_2 treated milk samples went through decolorization at different time intervals. Milk sample treated with 0.14% H_2O_2 was decolorized by 5 h later than the control one. Concentrations of H_2O_2 influence the color reduction time with an increasing manner where 0.02% and 0.04% H_2O_2 treated milk samples took the same time to decolorize (9 h). From this test, it is manifested that inhabiting properties of H_2O_2 retards the bacterial growth in treated milk samples which results in delaying decolorization. In agreement with the study Saha et al. (2003) reported that color reduction time of methylene blue test was consequently more for successive treatments (0.01, 0.02, 0.03, 0.04, 0.05 and 0.06%) except control. Saad et al. (2013) reported significant (P≤0.001) differences in microbial population by LP enzyme. McLay et al. (2002) used LP and monolaurin as anti-microbial agents and reported that LP system is considered to have greatest activity against Gram-negative bacteria. Touch et al. (2004) used LP system to

retard *Salmonella enteritidis* through catalyzing the oxidation of thiocyanate by H_2O_2.

CONCLUSION

In our country due to high temperature and humidity milk losses its shelf-life very quickly. Hydrogen peroxide is an effective chemical preservative for milk as it plays an important role in LP system. H_2O_2 is naturally present in milk but at lower concentration. After milking, the LP system persists in milk for very short length of time. If H_2O_2 is added to milk immediately after milking, this boosts the activity of LP system which ultimately prolongs the shelf-life of milk. In the present study, we added 10% H_2O_2 to milk at 0.02 to 0.14% (at 0.02% interval). At the room temperature it was found that the increased level of H_2O_2 results in delay in sour flavor development, positive COB test, decolorization of methylene blue and acidity development. Therefore, 0.14% H_2O_2 can be added to milk to preserve its consumption fitness, however further study is necessary considering the availability of SCN^- concentration in raw milk and residual concentration of H_2O_2 in milk after processing (pasteurization etc.).

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CONFLICT OF INTEREST

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

AUTHORS' CONTRIBUTION

SA and MNI planned and implemented study design. SA and MAHS carried out the experiment, collected and analyzed data, and finally prepared the manuscript. MAI and MHR critically checked and corrected the manuscript. MNI supervised the whole study with valuable suggestions.

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